



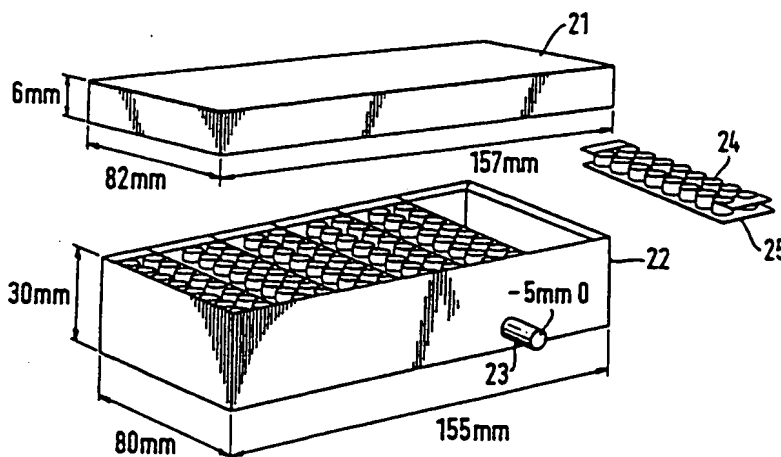
PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁶ : G01N 33/543, C12N 5/00</p>	<p>A1</p>	<p>(11) International Publication Number: WO 95/24648 (43) International Publication Date: 14 September 1995 (14.09.95)</p>
<p>(21) International Application Number: PCT/NO95/00052 (22) International Filing Date: 10 March 1995 (10.03.95) (30) Priority Data: 940866 10 March 1994 (10.03.94) NO (71)(72) Applicant and Inventor: FODSTAD, Øystein [NO/NO]; Frits Kizers vei 28, N-0383 Oslo (NO). (72) Inventors; and (75) Inventors/Applicants (for US only): HØIFØDT, Hanne, Kleppe [NO/NO]; Hvalstadåsen 25, N-1364 Hvalstad (NO). RYE, Philip, D. [GB/NO]; Melumveien 52, N-0751 Oslo (NO). (74) Agent: ONSAGERS PATENTKONTOR A/S; P.O. Box 265 Sentrum, N-0103 Oslo (NO).</p>	<p>(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published With international search report.</p>	

(54) Title: METHOD AND DEVICE FOR DETECTION OF SPECIFIC TARGET CELLS IN SPECIALIZED OR MIXED CELL POPULATIONS AND SOLUTIONS CONTAINING MIXED CELL POPULATIONS



(57) Abstract

The invention relates to a method and apparatus for detecting specific target cells in a simple and time-saving way, using paramagnetic particles, antibodies recognizing the Fc portions of target-cell associating antibodies and target-cell associating antibodies directed to specific antigen determinants in the target-cell membranes. Incubation of the cell suspension with detergent and/or second antibodies or antibody fragments, prelabeled or not with fluorescent agents, metalcolloids, radioisotopes, biotin complexes or certain enzymes allowing visualization, dramatically increase the specificity of the method. The method and apparatus described provides a solid support and permanent record which is easily viewed by microscopy, permits viewing and quantification of the whole specimen rather than small fractions thereof and allows the use of large specimen volumes to be analysed, the device may also be scanned automatically by conventional densitometric technology. The method and apparatus can be used for isolation of the target cells by magnetic field application, and a kit and apparatus for performing the method according to the invention is described.

Method and device for detection of specific target cells in specialized or mixed cell populations and solutions containing mixed cell populations

The present invention relates to an immunomagnetic method for detection of specific target cells in cell populations and solutions of cell populations. The invention also
5 relates to a kit and apparatus for performing the method in different cell populations.

In biology, biochemistry and adjacent fields there is considerable need for methods in which chemical entities are linked together. Such methods have an increasing importance in research regarding both normal and abnormal cell populations. Especially when solid supports are used cells can be immobilized, detected and
10 isolated and purified. Furthermore, the cell content may be examined using a range of sophisticated methods. It is also of importance to be able to isolate the cells in viable forms.

Affinity binding is a sophisticated way of linking chemical/biochemical entities together. In such a method a pair of binding partners, which for example are attached to the
15 substances to be linked, bind to each other when brought in contact. One of the binding partners in such a linkage may be represented by a molecule on the cell surface. Several such binding partner systems are known, such as antigen-antibody, enzyme-receptor, ligand-receptor interactions on cells and biotin-avidin binding, of which antigen-antibody binding is most frequently used.

20 When such methods are used for isolation of specific cells, which are the subject for further various examinations it is necessary that the cells should recover their function upon returning to the original conditions. This is not always the case, although it is proposed a method for providing physiological conditions such that the isolated specific cells can develop in sufficient numbers to allow further characterisation.

25 Methods are known in which one of the binding partners is attached to an insoluble support, such as paramagnetic particles, and by which isolation of target cells in a mixed cell population is performed as negative isolation or positive isolation. In a negative isolation procedure the unwanted cells can be removed from the cell preparation by incubating the cells with antibody-coated particles, specific for the
30 unwanted cells. Following the incubation the cell/antibody/particle complex can be removed using the particles, leaving the wanted target cells behind. This result is often not satisfactory, since the wanted cells are left in the cell population, more or less purified, and also since the intention of the isolation procedure is to examine and/or

perform further studies on the specific target cells. Attempts have been made to use particles for positive isolation, in which the wanted target cells are removed from the mixed cell population. These procedures have, however, been directed to certain target cells and are not suited for all target cell systems. A positive isolation procedure involving the hapten/anti-hapten linkage system has recently been proposed (WO91/01368) and relates to a method of connecting target cells to an insoluble support by using the abilities of hapten, antihapten antibodies and anti-cell antibodies to bind to each other, thus constructing a linkage between the insoluble support, i.e. particle, and the target cell, consisting at least of hapten and anti-hapten antibody or combinations of hapten and anti-hapten antibodies and anti-anti-hapten antibodies or secondary anti-cell antibodies. The later cleavage of the complex is performed by again exposing it to hapten or hapten analogue. Thus the constructed link always consists of hapten in addition to 1 or more elements. The method is directed to unspecified target cells and is directed to isolation of target cells and release of the insoluble support.

Furthermore, WO91/09938 describes the use of a combination of positive and negative selection for the purpose of isolating and possibly growing specific cells, i.e. haematopoietic progenitor cells, in the bone marrow, and is dependent upon removal of the particles. WO92/04961 comprises a method and a complicated equipment to separate cells or different molecules from a non-magnetic test medium by using colloidal magnetic particles. In this method small (sub micron) particles are used because it is necessary to avoid precipitation of the particles in the solution and this method necessitates complicated apparatus, in which magnetic intensifying means is immersed in the test medium. This may have adverse effects on the cells.

There is a need for a simple linkage to connect a target cell to an insoluble support, which does not involve compounds of a rather unspecified hapten-group, and which is directed to detection of specific target cells, with a minimum of unspecific cell association and which render unnecessary a later cleavage between the insoluble support and the specific target cell.

In a co-pending application by one of the applicants (WO94/07139, filed 10 September 1993) a method is described for detecting diagnostic purposes specific target cells without the problem with unspecific binding to normal cells. They represent sensitive detection methods for a variety of cell types, such that a high number of cells can be readily screened in the microscope and the procedure is rapid and simple. Furthermore, the methods can be used for isolation of cells for biochemical,

biological and immunological examination, and for studying of specific genes at the nucleotide or protein level, in addition to culturing the cells, without the need for cleaving the cell-particles complex. There is, however, a need for improvements such as isolation of the particle-bound target cells in the target cell suspension, from unbound beads, ~~unspecificall~~ bound non-target cells and unbound non-target cells, which is simple to perform, not time requiring and with render the target cell/particle complexes suitable to perform further analysis such as for example microscopic examinations and growing in a culture medium.

These objects are obtained by the present invention outlined by the method, apparatus and kit characterised in the enclosed claims.

The method for immunomagnetic detection of target cells in a mixed cell population and physiological solutions containing cell populations is suitable for detection, but may also be used in positive isolation of specific types of both normal cells and pathogenic cells. The method creates a linkage between a specific target cell and an insoluble support, such as paramagnetic particles, which consists of one or two elements. The particle is either coated with an anti-cell antibody of murine or human origin, directed to the specific antigen determinants in the membranes of the wanted target-cells, or the particles are coated with a polyclonal anti-mouse or anti-human antibody capable of binding to the Fc-portions of the specific anti-cell antibody directed to the antigen determinants in the target-cell membranes. Instead of using the polyclonal anti-mouse/anti-human antibody for coating the particles, a monoclonal rat anti-mouse/anti-human antibody may be used. This last antibody, due partly to its monoclonal origin, may provide a more specific binding to the anti-cell antibody and reduce the risk for possible cross-reactions with other cells in solutions, such as blood. Furthermore, incubation of the cell suspension with a mild detergent and/or second set of antibodies or antibody fragments, prelabeled or not with fluorescent agents, metalcolloids, radioisotopes, biotin-complexes or certain enzymes allowing visualization, will dramatically increase the specificity of the method.

Furthermore, according to the present invention, the method can be profoundly improved and simplified by transferring the suspension of target cell/particle complexes to the cell filtering device or cell separator according the present invention and the total number of target cells viewed microscopically or grown in a physiologically base culture medium to be characterised for the presence of specific biochemical and biological features.

Of the drawings:

Figure 1.1. shows a perspective view of an embodiment of the cell filtering device or Cell Separator, partly assembled.

5 Figure 1.2. shows a perspective view of another embodiment of the Cell Separator, partly assembled.

Figure 2. shows a perspective view of a version of the Cell Separator Multiwells with Membrane Filter detached from the Multiwells.

Figure 4. shows a perspective view of a version of the culture dish with lid arrangement for the Cell Separator Multiwells and/or the Membrane Filter.

10 Figure 4a. shows a side elevation of the Multiwell arrangement in the culture dish.

Figure 5. shows a perspective view of a version of the Cell Separator Filtrate Collection Box.

Figure 6. show melanoma cell-particle rosettes entrapped on cell filter device using the method described.

15 In the following a more detailed disclosure of the method is presented, using cancer cells as the target-cells for detection and possible isolation. The method is, however, not limited to cancer cells and the disclosure shall not limit the method to this particular field of use, since the method is suitable within a range of cytological research areas.

20 In the management of cancer patients, the staging of the disease with regards to whether it is localized or if metastatic spread has occurred to other tissues, is of utmost importance for the choice of therapeutic alternative for the individual patient. Malignant cells spread by direct invasion into the surrounding tissue, through the lymphatics or by the distribution of tumor cells in the blood to distant organs, including
25 the bone marrow and the central nervous system and the cerebrospinal fluid.

Detection of metastatic tumor cells has, until recently, relied on morphological methods using light and electron microscopy on biopsied tumor specimens, on smears of bone marrow and peripheral blood, and on slides prepared after cyto-centrifugation of various body fluids. Since the advent of monoclonal antibodies recognising antigens
30 predominantly expressed on the surface of different types of malignant cells, the identification of metastatic cells has, to an increasing extent, also involved immunocytochemistry and immunofluorescence. Thus, slides prepared from biopsied tumors or cyto-centrifugation have been treated with monoclonal antibodies, and the binding of these to the tumor cells is visualized colorimetrically or by fluorescence.
35 The latter method requires the use of a fluorescence microscope, alternatively

preparing a cell suspension and use of a flow cytometer.

The previous methods suffer from limited sensitivity and/or specificity, and is usually laborious and time consuming, also requiring a high degree of expertise. Flow cytometric examinations also involve expensive equipment.

5 The morphological methods for the detection of tumor cells in blood and bone marrow are much less sensitive than methods involving immunocytochemistry and immunofluorescence. Also the latter methods are, however, inadequate in cases where the tumor cells represent less than 1 % of the total number of nucleated cells. Flow cytometry may provide better sensitivity than the methods involving the use of a
10 microscope, but requires the availability of a high number of cells, and also involves several technical difficulties. Thus, aggregation of cells may cause problems, and the method does not provide possibilities to distinguish between labeled tumor cells and unspecifically fluorescing normal cells.

The invention allows for a very sensitive detection of, for example, metastatic tumor
15 cells, since a large volume and high number of cells can readily be screened in the microscope and the attached magnetic beads are easily recognisable. The method and apparatus described provides a solid support and permanent record which is easily viewed by microscopy, permits assessment and quantification of the whole specimen rather than small fractions thereof and allows the use of large specimen
20 volumes to be analysed, the device may also be scanned automatically by conventional densitometric technology. The monoclonal antibodies used bind with sufficient specificity to, for example, tumor cells and not to other cells than the target cells present in mixed cell suspensions, like blood, bone marrow, and in other tumor manifestations, such that all cells with attached beads represent the target-cells. In
25 addition, the procedure is rapid and simple, and can be performed by any investigator with access to a conventional microscope.

The novel method involves the binding of monoclonal antibodies, e.g. of murine or human origin, that specifically recognize antigens present on tumor cells, and not on
30 normal cells, to paramagnetic particles, either directly or to beads first covered with antibodies specifically recognizing the respective antibodies, or the Fc-portion of IgG antibodies, that bind to the tumor cells. The cell binding antibodies may be of the IgG or IgM type or being a fragment of ab IgG or IgM. Examples of used anti-target-cell antibodies may be those directed against groups of antigen determinants, for example

CD56/NCAM antigen (MOC-1), Cluster 2 epithelial antigen (MOC31), Cluster 2 (MW-40kD) antigen (NrLuLO) (Myklebust et al. Br. J. Cancer Suppl. 63, 49-53, 1991), HMW-melanoma-associated antigen (9.2, 27) (Morgan et al., Hybridoma, 1, 27-36, 1981), 80kD, Sarcoma-associated antigen (TP1 & TP3) (Cancer Res. 48, 5302-5309, 1988), mucin antigens (Diel et al., Breast Cancer Res. Treatm., 1991), or EGF-receptor antigen (425.3) (Merck), in addition to the anti-pan-human antibody (Bruland et al., unpublished), which is suitable for detecting human cells among animal cells. The 425.3 antibody is directed towards antigens in both normal and malignant cells. Antibodies can furthermore be directed against growth factor receptors, for example EGF-receptor, PDGF (A and B) receptor, insulin receptor, insulin-like receptor, transferrin receptor, NGF and FGF receptors, group of integrins, other adhesion membrane molecules and MDR proteins in both normal cells and abnormal cells, and antigens present on subpopulations of normal cells, in addition to oncogenic products, expressed on the membranes of normal and malignant cells and on malignant cells alone, for example Neu/erb B2/HER2. As for the malignant cells, these may be breast, ovarian and lung carcinoma cells, melanoma, sarcoma, glioblastoma, cancer cells of the gastrointestinal tract and the reticuloendothelial system, or the target-cells may be associated with non-neoplastic diseases, such as cardiovascular, neurological, pulmonary, autoimmune, gastrointestinal, genitourinary, reticuloendothelial and other disorders. Furthermore, the malignant cell population may be located in bone marrow, peripheral blood, come from pleural and peritoneal effusions and other body fluid compartments, such as urine, cerebrospinal fluid, semen, lymph or from solid tumors in normal tissues and organs, for example liver, lymph nodes, spleen, lung, pancreas, bone tissue, the central nervous system, prostatic gland, skin and mucous membranes. A more complete list of the antigen determinants and the corresponding antibodies or antibody fragments used in the present improved method is presented in Table 1 (see Appendix).

METHODOLOGY

The method comprises attachment of the antibodies directly to the paramagnetic particles, or the attachment can take place by attachment to surface-bound antibodies, such as polyclonal anti-mouse antibodies, monoclonal rat anti-mouse antibodies or monoclonal anti-human antibodies, specifically recognizing the Fc-portion of the said individual antibodies. The antibodycoated paramagnetic beads are then mixed with the suspension of cells to be examined and incubated for 5-10 min to 2 h, preferably for 30 min at 0-25C, preferably at 4C, under gentle rotation. The present method may also be performed in a changed order of steps, in that the free target-cell antibodies

are added to the cell suspension, incubated for 5-10 min to 2h, preferably 30 min, at 0-20C, preferably 4C, under gentle rotation. The paramagnetic particles, precoated with anti-mouse or anti-human antibodies are then added to the incubated cell suspension, as described above, and the resulting suspension subjected to a further incubation of 5-10 min to 2h, preferably 30 min, at 0-25C, preferably 4C under gentle agitation. The present method may also be performed in an abbreviated number of steps, in that the free target-cell antibodies are added to the cell suspension, at the same time and together with the precoated paramagnetic particles and subjected to incubation of 5-10 min to 2h, preferably 30 min, at 0-25C, preferably 4C under gentle agitation. The number of antibody-coated beads added to the cell suspension should be between 0.5-10 times the number of target cells. When this number is unknown, the amount of coated beads added should be 1-10 % of the total number of cells. For specific purposes, and in the cases where the density of the target-cells is low, for example malignant cells, or the target-cells represent a very low fraction of the total number of cells (about 1%), the target cells can be positively separated from non-target cells in a magnetic field. The isolated target cells, in cell suspension may then be transferred to a cell counting device, and the number of cells with attached beads may be determined by microscopy. The present method may also be performed, and preferably so, by transferring the isolated target cell suspension to the cell filtering device described in this application, and the total number of isolated target cells viewed by microscopy. The isolated target cells in the filter device may be fixed and stained to facilitate viewing by light microscopy. For specific purposes and in cases where the isolated target cells are required to be functionally active, a physiologically based culture medium may be added to the cell filter device and subjected to incubation for an unspecified time at 37C. The isolated target-cells may be grown and subsequently characterised for the presence of specific biochemical and biological features. Moreover, the target-cells may be characterised for the presence of specific biochemical and biological features. Of particular importance will be the use of such cells for studies in molecular biology. In contrast to the above cited methods of the prior art, the present method allows studies and growth of the target-cells without performing a cleavage of the paramagnetic particle target cell linkage. For several purposes it is of interest to examine specific genes in a pure population of target cells at the DNA, mRNA and protein level, both in tumor biopsies as well as in tumor cells present in blood, bone marrow and other body fluids, for example urine, cerebrospinal fluid, semen, lymph, or from otherwise normal tissues and organs, for example liver, lymph nodes, spleen, lung, pancreas, bone tissues, central nervous system, prostatic gland, skin and mucous membranes, and in other areas of cytological research activity. With the methods of prior art, signals obtained on Southern, Northern and

- Western blots represent the normal cells as well as the tumor cells in the biopsy. If a single cell suspension is first prepared from the tumor material, and the tumor cells are then positively immunomagnetically detected and separated, any gene studies performed on this material would represent the target-cells only. This also relates to
- 5 for example malignant cells present in mammalian tissues, for example in bone marrow, peripheral blood, pleural and peritoneal effusions, and other body fluids, for example urine, cerebrospinal fluid, semen and lymph. Studies involving polymerase chain reaction (PCR) methodology will also gain in specificity and reliability when performed on pure tumor cell populations obtained by the new method.
- 10 The application of the new method steps may differ depending on type of tissues to be examined.
- a) Tissue from solid or needle tumor biopsies is prepared mechanically or with mild enzymatic treatment into a single cell suspension, to which the primary, specific antibodies or antibody fragments are added directly or after washing the cell
- 15 suspension with phosphate buffered saline or culture medium with or without serum, such as fetal calf serum, bovine, horse, pig, goat or human serum.
- b) If the material is a sample of pleural or ascitic effusion, cerebrospinal fluid, urine, lymph or body fluids such as effusions in the joints of patients with various forms of arthritis, the specific antibodies or antibody fragments are either added to the samples
- 20 directly, or after centrifugation with or without washings before or after the cells in the samples are spun down and brought back into suspension.
- c) If the material consists of blood or bone marrow aspirate, the specific antibodies or antibody fragments are either added to the samples directly, or after centrifugation with or without washings before or after the cells in the samples are spun down and
- 25 brought back into suspension, or a mononuclear cell fraction may be prepared by gradient centrifugation on e.g. Lymphoprep before washing, resuspension, and addition of the appropriate antibodies or antibody fragments.
- The procedure conditions for a) and b) are established, as exemplified by results obtained in successful experiments as those described below.
- 30 For c) the results have been found to be influenced by a high number of factors which have been examined in detail. Among these are antibody concentration, the ratio of the number of paramagnetic particles versus number of cells, incubation times and

volumes, type of incubation medium, and the pH level. The particle to mononuclear cell ratio in all experiments should be in the range of 0.5/1 - 2/1, depending on the binding affinity of the primary specific antibodies or fragments.

5 A major problem has been unspecific attachment to normal blood or bone marrow cells of particles coated with either sheep or rat anti-mouse antibodies alone, or in addition with the specific antibodies. Experiments have shown that the unspecific binding is equally high without the presence of the specific antibodies, indicating that the problem is not caused by crossreactivity of the targeting antibodies to normal cells. The possibility that the less than optimal specificity could be caused by ionic binding
10 has been ruled out. Another possibility was that subpopulations of normal cells of the B-lineage might adhere to the particle-antibody complexes. However, immunomagnetic removal of B-cells from the cell suspension before adding the specific antibodies/antibody-particle complexes did not improve the specificity of the latter.

15 The problem with the procedure used on isolated mononuclear fractions of bone marrow and peripheral blood, that some non-target cells might also bind paramagnetic particles, has been circumvented or overcome. Thus with sheep-anti-mouse antibody coated particles alone or with specific antibodies the number of particles unspecifically attached to a low fraction mononuclear blood or bone marrow cells was reduced from an average of 10 to about 1 and in parallel the fraction of normal cells with particles
20 decreased from 1-2% to 0.5-1% or less.

Evidence has been obtained that the problem may be caused by hydrophobic forces associated with the antibodies bound to the paramagnetic particles. Methods for reducing this hydrophobicity is thus claimed. One such method is pre-incubation of the antibody-coated particles and the cell suspension with ~~the~~ detergents in suitable
25 concentrations, for example Tween 20™ in concentrations of less than 0.1% for 30 minutes at 4C. When possible selection of the target cells is warranted, the cell suspension should contain a low concentration of the detergent, e.g. 0.01% of ~~Tween~~ 20™. In several experiments this procedure has almost eliminated or dramatically reduced the problem of unspecific binding seen with the mononuclear cell fractions
30 from blood or bone marrow.

The other improvement which, if found warranted, may be used together with the detergent step as follows:

After incubation of the cell suspension with the primary antibodies or antibody

fragments and the antibody-coated paramagnetic particles as described in previously, the cell suspension is incubated with a second set of antibodies or antibody fragments directed against other extracellular or against intracellular determinants of the target cells, with or without pre-treatment with cell fixatives such as formaldehyde or alcohols. These antibodies or their fragments should have been prelabeled by fluorescent agents, metalcolloids, radioisotopes, biotin-complexes or enzymes like peroxidase and alkaline phosphatase, allowing visualization by *per se* known methods in the microscope and/or a suitable counting device.

The target cells will both be visualized with the latter method and have bound particles to their surface, and can thus be enumerated.

To simplify the distinction between non-target and target cells, the cell suspension, or part thereof, can before the second visualization step either be subjected to cytospin centrifugation or portions of the suspension are attached to coated glass slides on which the particle-bound cells will be spread out in a thin layer, facilitating the recognition of the double-"stained" cells.

An alternative method according to the present invention to further simplify the distinction between non-target and target cells comprises the cell filter device, wherein the whole cell suspension after the target cell selection steps, can be added directly to the cell filter device. The free unbound beads, unspecifically bound non-target cells, and any unbound non-target cells, will pass through the filter leaving the bound target cells to be visualized on the filter. The filter with the isolated target cells can be removed from the device and the cells may be fixed and stained using known immunohistochemical methods and viewed by microscopy. After the filter has been removed from the device it can be treated as a conventional microscope slide of the type that is known and normally used in immunohistochemistry.

For specific purposes the filter may either be removed from the device or remain integral to the device, and a culture medium added, such as any known culture medium with or without agarose, for the purpose of propagating the isolated target cells situated on the filter.

For use in the new procedure, kits will contain for example precoated paramagnetic particles prepared for each monoclonal antibody. In another embodiment the kits contain paramagnetic particles pre-coated with IgG isotype specific anti-mouse or anti-human antibody as one part of it, and different target cell-associated, for example

tumor cell, antibodies as another part. In a third embodiment the kit contains paramagnetic particles precoated with specific anti-Fc antibodies, such as polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-mouse, or anti-human antibodies, capable of binding to the Fc portion the target-cell associating antibodies, bound to
5 specific anti-target-cell antibodies. In a fourth embodiment the kits contain distinctive particles of a paramagnetic or non-magnetic nature, which may be coated or uncoated with a target-cell antigen or group of target-cell antigens, such that when processed by the method these particles become entrapped in the cell filter device, thereby acting as a control in demonstrating for example that all aspects of the antibody-antigen
10 interactions in the method are working correctly. These particles may be incorporated into the cell suspension at a stage before or during the method, or the particles may be used as a separate "cell suspension" to be processed using the same method as the cell suspension comprising the target cells to be separated. In a further embodiment the kit contains other specific antibodies or antibody fragments directed
15 against antigens/receptors within or on the wanted target-cells, where said antibodies or antibody fragments are conjugated to peroxidase, alkaline phosphatase, or other enzymes, together with relevant substrates to such enzymes, or where said antibody or antibody fragment is bound to non-paramagnetic particles with specific colours or with bound enzymes such as peroxidase and alkaline phosphatase.

20 APPARATUS

The new feature of the method concerns a cell filter device, which may also be termed a multiwell cell separator, and may or may not be a part of the kit as described. The device concerns a microwell cell separator arrangement, which is used to separate mixed populations of different sized cells, such as those found in blood or
25 bone marrow. The resulting cells can be viewed directly on the membrane by microscopy or automated scanning devices. This invention may be used in conjunction with conventional magnetic particle cell isolation techniques to provide a rapid, sensitive, and simple method for screening large numbers of high or low volume samples for the presence of tumour cells within 1 to 4 hours.

30 According to the present invention there is provided a microwell cell separator arrangement comprising an open topped filtrate collection box, which may or may not have an attachment for a vacuum tube, and has a removable and disposable multiple wells arrangement with a cell separating membrane filter which forms the base of these multiple wells. A lid or cover to this arrangement may also be provided for.

The filtrate collection box and lid arrangement may be made from a material suitable for high temperature sterilisation, or may be made from a plastic transparent or opaque plastic material such as is known for tissue culture plastic wares.

5 The cell separating membrane filter may comprise a regular and consistent pore shape and size, such as is found in nylon monofilament membranes, which forms the base of the individual wells. The cell separating membrane filter may be secured to the microwells such that it can be removed after the cell separation method in order to facilitate examination. The cell separating membrane may also comprise a card or plastic surrounding frame to facilitate examination after removal from the microwells.

10 The filtrate collection box may comprise a frame in which removable strips of more than one well may be inserted.

The filtrate collection box may be fashioned similar to a conventional 96-well plate adapted to accommodate the cell separating membrane, collection box and low pressure vacuum attachment.

15 The invention may also comprise an upper lid or cover.

A disposable culture dish with lid is provided for in the device that allows the microwell strips to be inserted and cultured aseptically. Integral to the culture dish are indentations or notches that facilitate the positioning of the microwell strip similar to that in the filtrate collection box, and to prevent movement of the microwell strip during culture. The indentations or notches as described may or may not also provide for the location of the cell separating membrane after removal from the microwell strip.

20

The invention will be further apparent from the following description with reference to the figures of the accompanying drawings, which show, by way of example only, one form of the microwell cell separator arrangement embodying the same.

25 Referring to Figures 1 to 5 of the drawings it will be seen that the Microwell Cell Separator arrangement 20 consists of a lid or cover 21 and a filtrate collection box 22, which may or may not have a low pressure vacuum attachment port 23, with removable Multiwell strips 24 which have a detachable membrane base 25 with support 25a.

30 Figure 1.1. and 1.2. shows two alternative embodiments of the invention partially

assembled.

The filtrate collection box 22 may be similar in some respects to conventional 96-well plate formats with removable well strips, and may be arranged to fit one or multiple strips of wells.

- 5 The Multiwells 24 may be arranged in double strips as shown or in single or multiple strips.

The engagement of the Multiwells 24 in the Filtrate Collection Box 22 is such that only one orientation is possible, which may be provided for by locating pins 28 or notches 29.

- 10 The Cell Separator Membrane Filter 25 is fixed to the bottom of the Multiwells 24 and forms the base of the wells. The fixing of the membrane filter 25 to the Multiwells 24 is such that they can be separated without deformation of the membrane filter 25 or the membrane filter support 25a.

- 15 The membrane filter 25 can be viewed by microscopy or may be scanned by densitometric or similar methodology.

The membrane filter 25 may comprise a regular and consistent pore shape and size, such as is found in nylon monofilament membranes, which forms the base of the individual wells, and may be of 5-75m pore size but preferably 20m.

- 20 The Multiwells 24 within or without the Filtrate Collection Box 22 may also be made of a material suitable for tissue culture purposes, which may also be suitable for viewing in conventional 96-well plate scanning or plate reading machines.

The culture dish 26 and culture dish lid 27 may also be made of a material suitable for tissue culture purposes. In this way it is possible to supply culture medium both through the top of the multiwells and in the bottom of the culture dish 26.

- 25 All dimensions shown in the figures are exemplary and the cell filtering device 20 should not be limited by these dimensions. Furthermore, it will be appreciated that it is not intended to limit the invention to the above example only, many variations being possible without departing from the scope thereof. The present method will in the following be illustrated by model experiments, examples of the usefulness of the new

method and examples of practical applications. These examples shall not be regarded as in any way limiting the invention.

MODEL EXPERIMENTS

- 5
10
15
20
25
30
1. *Binding of antibody-bead complexes to tumor cell lines.* To determine antibody concentrations and optimal conditions for the binding of antibody-paramagnetic particle complexes to tumor cells, a large panel of cancer cell lines was used. The paramagnetic beads were bound to the cells, either by coating the specific antibodies to sheep-anti-mouse antibody (SAM)-coated paramagnetic particles, or by first incubating the cells with the specific antibodies, washing, followed by a second incubation with SAM-coated particles. The results of these experiments are given in Tables 2a and 2b (see Appendix), in which + indicates binding of several beads to all cells, (+) indicates either a lower number of beads bound to each cell, or that not all the tumor cells had beads attached to their surface, whereas reflects no binding, and (-) indicates very weak binding.
2. *Detection of tumor cells in the mononuclear fraction of bone marrow or peripheral blood.* Model experiments were performed where specific antibodies and SAM-coated paramagnetic particles were added either to such mononuclear cells or to a cell suspension where a different number of cancer cells from in vitro cultivated cell lines were added to said mononuclear cells. In some experiments, either the mononuclear cells, or the malignant cells were prestained with a fluorescent dye, to be able to distinguish between the two types of cells. In all experiments, non-binding primary antibodies, and/or sheep-anti-mouse antibody-coated beads were used separately as controls. Additional experiments without the preparation of a mononuclear cell fraction of peripheral blood were performed. It was found that the separation of cells in this way reduced the amount of unspecific binding compared to the Lymphoprep separated blood fractions.
3. *Separation and visualisation of antibody-bead complexes to tumor cell lines using the cell filter device.* The tumor cell suspensions and fluorescent labelled tumor cells mixed with blood or bone marrow suspensions were prepared and treated as described in the model experiments 1. and 2., and were subjected to the cell filter device. After washing, fixing and staining the cells on the filter in the device the filter was viewed by microscopy. The results from the tumor cell suspension alone showed antibody-bead-tumor cell complexes clearly isolated on the filter. The results from the fluorescent labelled tumor cell suspension together with blood or

bone marrow also showed antibody-bead-tumor cell complexes clearly isolated on the filter (Figure 6). Additional experiments to test the sensitivity of the method showed that 100 tumor cells, when mixed with a suspension of 10^7 blood or bone marrow cells, could be detected using this method.

- 5 4. *Growth of separated cells isolated using the cell filter device.* Tumor cell suspensions treated and isolated as described in the model experiments 1. and 2., were subjected to the filter device and the filter was subsequently incubated in a semi-solid medium containing 0.3% agarose in culture medium containing 20% calf serum. The cells were incubated in an
10 atmosphere of 5% CO_2 at 37C. The tumor cells showed an ability to divide and grow.

In several experiments some unspecific binding to the mononuclear cells was observed, which was found to be unrelated to the nature of the specific antibody, and which was equally pronounced with SAM-coated particles alone.
15 The magnitude of this unspecific binding varied from almost 0% to a level between 0.5-2%. This unspecific binding was almost eliminated by mild treatment with detergent, (Tween 20TM) performed to reduce the problem of hydrophobic cell interactions.

EXAMPLES OF THE USEFULNESS OF THE PROCEDURE

- 20 1. *Detection of micrometastatic neoplastic disease in blood and marrow.* Early and reliable diagnosis of spread of cancer cells to blood and/or bone marrow has become increasingly important for the choice of optimal therapy, possibly curative in many types of cancer, including carcinomas, as described in application Example 1. Similar procedures for malignant
25 melanoma, sarcoma, neuroblastoma and several other cancers have been established or are under development.
- 30 2. *Detection of malignant cells in pleural or ascitic effusions and urine.* The nature of such effusions may represent an important diagnostic problem, particularly when a low number of cancer cells are present together with normal reactive or epithelial cells. In several cases a definite diagnosis has been rapidly made with the new method, in cases where conventional
cytological examination has been negative or inconclusive. A similar advantage can be found in cases of cancer in the kidneys or in the urinary

tract and bladder.

3. *Detection of neoplastic cells in the cerebrospinal fluid.* As the systemic treatment of many cancer types have improved, the frequency of cases with symptom-giving brain metastases have significantly increased, and in parallel with this, the necessity for early detection of such spread. With the use of the new procedure even a low number of malignant cells can easily be identified, permitting intervention with therapeutic alternatives at an early stage of intracranial tumor manifestations.
4. *Diagnosis of cancer in biopsied tissue.* When cancer is suspected, and tissue biopsies are obtained by surgical procedures or by e.g. needle biopsies, a much more simple and rapid diagnosis can be made with the new method, used on prepared cell suspensions. compared to conventional morphological or immunohistochemical or cytochemical procedures. Distinction between several alternative cancers can be made by the use of the appropriate antibodies.
5. *Identification of prognostic indicators.* Since the expression of several membrane molecules have been shown to correlate with progression of the malignant disease in several cancers, the present method can be used to identify prognostic indicators, for example as described in application Example 2.
6. *Identification of cells indicative of specific diseases or of disease progression or state.* In various types of rheumatoid diseases (such as rheumatoid arthritis), as well as in allergic, autoimmune, and cardiovascular diseases, identification of the systemic or local presence of specific subpopulations of cells is important for diagnosis and for determining the stage of the disease. Rapid detection of such cell populations with the new method is therefore of considerable diagnostic and therapeutic importance.
7. *Detection of subpopulations of normal cells.* For several purposes, it will be important to detect the fraction of a particular subpopulation of normal cells in a population. This applies e.g. to liver biopsies where the identification of cells expressing the biliar epithelial antigen, may be of importance. Similarly, the identification, and possible isolation of specific endothelial cells from a cell suspension prepared from various normal tissues may be

warranted.

8. *Isolation and growth of selected cells.* For many of the above mentioned purposes it may be required to have a larger population of cells to study. The present method using the cell filter device can provide the conditions to permit the propagation of the positively selected target cells, without the presence of free unbound particles or other unspecifically bound cells.

Several of the cell membrane molecules mentioned above in sections 1-6 may also be used as targets for immunotherapy with several types of activated killer cells or for example with immunotoxins. The identification with the new method of expression of such molecules is, therefore, also of value for determining in which cases such types of therapy should be used.

EXAMPLES OF A PRACTICAL APPLICATION OF THE METHOD

Example 1. To diagnose spread of cancer cells in blood and/or bone marrow at an early stage, we have used in the new procedure the MOC-31, NrLul0, BM2, BM7, 12H12, and MLuCl anti-carcinoma antibodies to determine whether or not micrometastatic disease from breast, lung, colorectal, and prostate cancer might be sensitively identified in such body fluids. The successful results with these antibodies have significant clinical implications.

Example 2. The expression of many cell membrane molecules have been shown to correlate with progression of the malignant disease in several types of cancer. The detection of binding of such molecules to respective antibodies can therefore be used to obtain information of high prognostic value. Among such antigens are a high number of adhesion molecules, carbohydrate antigens, glycolipids, growth factor receptors and carcinoma markers listed below. We have, with the new procedure identified the binding of particle-antibody complexes to CD44 variants, E-cadherin, Le^x, CEA, EGF-r, transferrin receptor, MUC-1 epitope, LUBCRU-G7 epitope, prostate cancer antigen, UJ13A epitope, 2-microglobulin, HLA-antigens, and apoptosis receptor.

Example 3. Two litres of pleural effusion from a patient supposed to suffer from malignant melanoma was obtained. After centrifugation, the cells were suspended in a volume of 2 ml of RPMI with a 10% fetal calf serum, incubated with 9.2.27 anti-melanoma antibody (10 g/ml) at 4C for 30 min,

washed and again incubated with Dynabeads™ SAM M450/IgG2A at 4C for 30 min. The cell suspension was then examined under a microscope for determining the fraction of cells with paramagnetic cells attached to their surface. The diagnosis of malignant melanoma was confirmed, as about 10% of the cells had a significant number of bound particle-rosettes.

Example 4. Biopsied tissue was obtained from a subcutaneous tumor in a case with clinical indications of either small cell lung cancer or a malignant melanoma. A single cell suspension was prepared from the biopsy, divided in 2 fractions, one incubated with the 9.2.27 anti-melanoma antibody, and the other with MOC-31 anti-carcinoma antibody (both at 10 g/ml). The incubation was similar to that used in the example above. None of the cells incubated with the melanoma antibody bound any beads, whereas all tumor cells incubated with MOC-31 were positive.

Example 5. Biopsied tissue from a patient suspected to have malignant melanoma was examined by preparing single cell suspension, incubating with 9.2.27 anti-melanoma antibody, and then following the procedure as above. Most of the cells were positive with a high number of particle-rosettes attached to their membranes.

Example 6. A pleural effusion from a breast cancer patient was studied to examine whether tumor cells could be detected in the fluid. One litre of the fluid was centrifuged, the cells resuspended, and in separate vials incubate with each of 3 different anticarcinoma antibodies (MOC-31, 2E11, 12H12). After completing the procedure as in the previous example, it was found that most of the cells bound to antibody-coated particles in all 3 cases.

Example 7. A bone marrow suspension obtained from a breast cancer patient was studied to examine whether micrometastatic tumor cells could be present. After the preparation of mononuclear cells, these were incubated with the same 3 anti-carcinoma antibodies used in the example above, but in this case the antibodies were first attached to Dynabeads™ SAM IgG paramagnetic particles. After 1 incubation with these directly coated particles, the cell suspension was examined in the microscope, and a high number of cells were found positive with a number of particle-rosettes attached to their membrane. Similar experiments have been performed in a number of pleural or ascitic effusion and bone marrow from patients with breast.

Example 8. T47D human breast carcinoma cells were incubated for varying lengths of time with Hoechst fluorescence dye, and the viability of the labeled cells was checked. Varying numbers of labeled breast carcinoma cells were then added to 1×10^6 bone marrow cells obtained from healthy volunteers. In
5 different experiments, different concentrations of paramagnetic, monodisperse particles (Dynabeads™ P450) coated with individual anticarcinoma antibodies (NrLu10, MOC31, or 12H12) were added. After incubation for 30 min on ice, samples of the different test tubes were examined in a counting chamber under light and fluorescence microscopy. When the ratio of tumor cells/total nucleated
10 cells was low, the cell suspension was subjected to a magnetic field and the cells with particles attached were isolated before examined in the microscope. It was found that at an optimal ratio of 1-10 paramagnetic beads per tumor cell in the cell mixture, all the tumor cells had from 2-15 beads attached to their surface. The sensitivity of the detection method was close to one target-cell per
15 10^4 nucleated cells. In control experiments with labeled tumor cells using antibodies known to have some cross-reactivity to normal cells, this cross-reactivity was confirmed with the antibody-coated paramagnetic particles. In experiments with beads without tumor-associated antibody coating, none of the target cells bound any beads.

20 Similar experiments have been performed both with other breast cancer lines and a small cell lung cancer cell line. Similar sensitivity and specificity were obtained in these experiments.

Example 9. Pleural and ascites fluid from patients with breast cancer and ovarian carcinoma were centrifuged, the same coated paramagnetic particles
25 used in Example 1 were added, incubated and concentrated in a magnetic field before the suspension was examined under light microscopy. Typically, cells that had the clear morphological features of tumor cells had beads attached, whereas none of the few normal cells bound the antibody-coated beads. In two cases with pleural effusion, an independent morphological examination did not
30 reveal the presence of any tumor cells, whereas a significant number malignant cells were detected by the use of antibody-coated beads. In some cases, tumor cells were separated in a magnetic field and transferred to tissue culture flasks containing growth medium specially prepared for growing breast cancer cells, in attempts to establish permanent cell lines from these cultures. In parallel, cells
35 from the malignant effusions were cultivated directly without positive selection with magnetic beads. In the latter cases, no cell line could be established,

whereas in more than 50 % of the cases where positively selected tumor cells had been used, cell lines were successfully established.

Example 10. In some cases, bone marrow and peripheral blood obtained from patients with breast cancer were examined with the present procedure by adding
5 antibody-coated paramagnetic beads, incubating for 30 min at 4C and concentrating in a magnetic field and by examining the suspension under light microscopy. In both cases binding of the paramagnetic beads to tumor cells, representing 0.1-1 % of the nucleated cells in the bone marrow and blood was detected, cells that could not be identified by any other method.

10 *Example 11.* Antibodies against certain growth factor receptors or other gene products expressed on the surface of specific cell populations may be used to identify and positively select these cells. Beads coated with anti-transferrin receptor antibodies, used in the novel method according to the present invention
15 were shown to represent a rapid, simple and sensitive method for identification of cells expressing the transferrin receptor.

Example 12. For various purposes isolation of specific populations of normal cells is warranted. Endothelial cells lining the capillary or small vessels in normal or tumorous tissue could be positively selected from cell suspensions prepared from the relevant tissues. The procedure involved the use of beads
20 coated with antibody directed against structures expressed on the endothelial cells, but not on the other normal cells in the cell mixture.

Example 13. Human cells injected into immunodeficient rodents was shown to be present in cell suspensions prepared from tumor xenografts and from various host organs/tissues by employing magnetic particles coated with an anti-pan
25 human antibody.

Example 14. Tumor cell lines from breast carcinoma and melanoma patients were separated from a mixed population of blood or bone marrow cells and filtered using the cell filter device described. After the addition of culture medium and subsequent incubation the selected tumor cells on the filter were
30 able to grow in the absence of free unbound particles or other unspecifically bound cells.

Table 1. List of relevant antigens and examples of associated antigen-binding antibodies

	ANTIGENS	MONOCLONAL ANTIBODIES
	Adhesion molecules	
5	Fibronectin receptor ($\alpha 5\beta 1$ integrin)	Pierce 36114, BTC21/22 Calbiochem 341649
	Integrin $\alpha 3\beta 1$	M-Kiol 2
	Vitronectin receptor ($\alpha v\beta 3$ integrin)	TP36.1, BTC 41/42
	Integrin $\alpha 2$	Calbiochem 407277
	Integrin $\alpha 3$	Calbiochem 407278
10	Integrin $\alpha 4$	Calbiochem 407279
	Integrin $\alpha 5$	Calbiochem 407280
	Integrin αV	Calbiochem 407281
	Integrin $\beta 2$	Calbiochem 407283
	Integrin $\beta 4$	Calbiochem 407284
15	GpII β III α	8221
	ICAM-I (CD54)	C57-60, CL203.4, RR 1/1 ¹
	VCAM-1	Genzyme 2137-01
	ELAM-1	Genzyme 2138-01
	E-selectin	BBA 8
20	P-selectin/GMP-140	BTC 71/72
	LFA-3 (CD58)	TS 2/9
	CD44	BM 1441 272, 25:32
	CD44-variants	11.24, 11.31, 11.10
	N-CAM (CD56)	MOC-1
25	H-CAM	BCA9
	L-CAM	BM 1441 892
	N-CAM	TURA-27
	MACAM-1	NKI-M9
	E-cadherin	BTC 111, HECD-1, 6F9
30	P-cadherin	NCC-CAD-299
	Tenascin	BM 1452 193 Calbiochem 580664
	Thrombospondin receptor (CD36)	BM 1441 264
	VLA-2	A1.43
	Laminin receptor	

	HNK-1 epitope	HNK-1
	Carbohydrate antigens	
	T-antigen	HH8, HT-8
	Tn-antigen	TKH6, BaGs2
5	Sialyl Tn	TKH-2
	Gastrointestinal cancer associated antigen (M _w 200kD)	CA 19-9
	Carcinoma associated antigen	C-50
	Le ^y	MLuCl, BR96, BR64
10	di-Le ^x , tri-Le ^x	B3
	Dimetric Le ^a epitope	NCC-ST-421
	H-type 2	B1
	CA15-3 epitope	CA15-3
	CEA	I-9, I-14, I-27, II-10, I-46
		Calbiochem 250729
15	Galb1-4GlcNac (nL4,6,8)	1B2
	H-II	BE2
	A type 3	HH8
	Lacto-N-fucopentannose III (CD15)	PM-81
	Glycolipids	
20	GD ₃	ME36.1, R24
	GD ₂	ME36.1, 3F8, 14.18
	Gb ₃	38-13
	GM ₃	M2590
	GM ₂	MKI-8, MKI-16
25	FucGM ₁	1D7, F12
	Growth factor receptors	
	EGF receptor	425.3.2.E9, 225
	c-erbB-2 (HER2)	BM 1378 988, 800 E6
	PDGFα receptor	Genzyme 1264-00
30	PDGFβ receptor	Sigma P 7679
	Transferrin receptor	OKT 9, D65.30
	NGF receptor	BM 1198 637
	IL-2 receptor (CD25)	BM 1295 802, BM 1361 937
	c-kit	BM 428 616, 14 A3, ID9.3D6

	TNF-receptor NGF-receptor	Genzyme 1995-01, PAL-M1
	Melanoma antigens	
5	High molecular weight antigen (HMW 250.000)	9.2.27, NrML5, 225.28 763.74, TP41,2, IND1
	Mw105 melanoma-associated glycoprotein	ME20
	100kDa antigen (melanoma/carcinoma)	376.96
	gp 113	MUC 18
10	p95-100	PAL-M2
	Sp75	15.75
	gr 100-107	NKI-bereb
	MAA	K9.2
	M ₁ 125kD (gp125)	Mab 436
15	Sarcoma antigens	
	TP-1 and TP-3 epitope	TP-1, TP-3
	M _w 200kD	29-13, 29.2
	M _w 160kD	35-16, 30-40
	Carcinoma markers	
20	MOC-31 epitope (cluster 2 epithelial antigen)	MOC-31, NrLu10
	MUC-1 antigens (such as DF3- epitope (gp290kD)	MUC-1, DF3.BCP-7 to -10
	MUC-2 and MUC-3	PMH1
25	LUBCRU-G7 epitope (gp 230kD)	LUBCRU-G7
	Prostate specific antigen	BM1276 972
	Prostate cancer antigen	E4-SF
	Prostate high molecular antigen M _w >400kD	PD41
30	Polymorphic epithelial mucins	BM-2, BM-7, 12-H-12
	Prostate specific membrane antigen (Cyt-356)	7E11-C5
	Human milk fat globulin	Immunotech HMFG-1, 27.1
	42kD breast carcinoma epitope	B/9189
35	M _w >10 ⁶ mucin	TAG-72, CC-49, CC-83

	Ovarian carcinoma OC125 epitope (M _w 750 kD)	OC125
	Pancreatic HMW glycoprotein	DU-PAN-2
	Colon antigen Co17-1A(M _w 37000)	17-1A
5	G9-epitope (colon carcinoma)	G9
	Human colonic sulfomucin	91.9H
	M _p 300kD pancreas antigen	MUSE11
	GA 733.2	GA733,KS1.4
	TAG 72	B72.3, CC49, CC83
10	Undefined	Oa11, SM1
	Pancreatic cancer-associated	MUSE 11
	Pancarcinoma	CC49
	Prostate adenocarcinoma-antigen	PD 41
	M _w 150-130kD adenocarcinoma of the lung	AF-10
15	gp 160 lung cancer antigen (Cancer Res. 48, 2768, 1988)	anti gp160
	M _w 92kD bladder carcinoma antigen	3G2-C6
	M _w 600kD bladder carcinoma antigen	C3
20	Bladder carcinoma antigen (Cancer Res. 49, 6720, 1989)	AN43, BB369
	CAR-3 epitope M _w >400kD	AR-3
	MAM-6 epitope (C15.3)	115D8
	High molecular ovarian cancer antigen	OVX1, OVX2
25	Mucin epitope Ia3	Ia3
	Hepatocellular carcinoma antigen	KM-2
	M _w 900kD	
	Hepemal epitope (gp43) Hepato-cellular carc. ag	Hepema-1
30	O-linked mucin containing N-glycolylneuraminic acid	3E1.2
	M _w 48kD colorectal carcinoma antigen	D612
35	M _w 71kD breast carcinoma antigen	BCA 227
	16.88 epitope (colorectal carcinoma-antigen)	16.88
	CAK1 (ovarian cancers)	K1

5	Colon specific antigen p Lung carcinoma antigen M _w 350-420kD gp54 bladder carcinoma antigen gp85 bladder carcinoma antigen gp25 bladder carcinoma antigen	Mu-1, Mu-2 DF-L1, DF-L2 T16 T43 T138
10	Neuroblastoma antigens Neuroblastoma-associated, such as UJ13A epitope Glioma antigens Mel-14 epitope	UJ13A Mel-14
15	Head and neck cancer antigens M _w 18-22kD antigen HLA-antigens HLA Class 1 HLA-A HLA-B HLA-A2 HLA-ABC	E48 TP25.99 VF19LL67 H2-149.1 KS1 W6.32
20	HLA-DR, DQ, DP β2-microglobulin Apoptosis receptor Apo-1 epitope	Q 5/13, B 8.11.2 NAMB-1 Apo 1
25	Various Plasminogen activator antigens and receptors p-glycoprotein cathepsin D biliary epithelial antigen	Rabbit polyclonal C219, MRK16.JSB-1, 265/F4 CIS-Diagnostici, Italy HEA 125
30	neuroglandular antigen (CD63) CD9 pan-human cell antigen	ME491, NKI-C3, LS62 TAPA-1, R2, SM23 pan-H

Table 2a. Results of antibody binding with different cell lines

Antibodies	Cell Line MCF-7	Cell line SKBR3	Cell line T47D	Cell line MDA231	Cell line MDA435	Cell line DU145	Cell line FEMX-1	Cell line LOX
NrLu10 IgG2b		+	+	(+)	(+)	+		
Moc31 IgG1	+	+	+	(+)	(+)	+		
Moc1 IgG1			(+)	(+)	+			
12H12 IgG1		+	+		+	+		
2E11 IgG3	+	+	+		+	+		
5A6 IgG1		(+)	+		+			
5F2 IgM			(+)					
CC3 IgG2a	-	-	-	-			(+)	
CC1 IgM	-	-	-	-				
CU18 IgG1	-	-	-	-				
CU46 IgG1	(+)	-	-	-				
7F11 IgG1	-	-	+	+				
ID7 IgG3			(+)					
E4SF IgG1		+	+	+		(-)		50%
425-3			+					-
9.2.27								-
MUC18		-					+	-
2g12 IgG1								
4b7							+	
IgG1	+		+				+	
BCRU-G7 IgM							+	

Table 2b. Results of antibody binding with different cell lines

Antibodies	Cell line PM1	Cell line MA-11	Cell line CRL-1435	Cell line CRL-1740	Cell line IL-146	Cell line Colo-205	Cell line 786-O	Cell line WIDr
NrLu10	+	+	+	+	+	+	-	+
IgG2b	+	+	+	+	+	+	+	+
IgG1	+	+	+	+	+	+	+	+
Moc31	+	+	+	+	+	+	+	+
Moc1	+	+	+	+	+	+	+	+
12H12	+	+	+	+	+	+	+	+
2E11	(+)	+	(+)	+	+	+	+	+
5A6	+	+	+	+	+	+	+	+
CC3	+	+	+	+	+	+	+	+
CC1	+	+	+	+	+	+	+	+
CU18	+	+	+	+	+	+	+	+
CU46	+	+	+	+	+	+	+	+
7F11	(+)	+	+	+	+	+	+	+
ID7	+	+	+	+	+	+	+	+
E4SF	+	+	+	+	+	+	+	+
MUC18	+	+	+	+	+	+	+	+
2g12	+	+	+	+	+	+	+	+
4b7	+	+	+	+	+	+	+	+
BM2 (=2F11)	+	+	+	+	+	+	+	+
BM7 (=7F11)	+	+	+	+	+	+	+	+
GINTES	+	+	+	+	+	+	+	+
3C9	+	+	+	+	+	+	+	+
11H8	+	+	+	+	+	+	+	+
5F4	+	+	+	+	+	+	+	+
3F1	+	+	+	+	+	+	+	+

SUBSTITUTE SHEET

PATENT CLAIMS

1. Method for detecting specific target cells in cell suspensions of mixed cell populations and in fluid systems containing mixed cell populations, and in single cell suspensions prepared from solid tissues, except normal and
5 ~~align haematopoietic cells in blood and bone marrow,~~
~~characterised by~~ comprising the following steps:
- 1.1. coating, by a *per se* known procedure, paramagnetic particles or beads with either, a) antibodies, or antibody fragments directed against
10 membrane structures specifically expressed on target-cells and not on non-target-cells in the cell mixture or; b) antibodies, preferably polyclonal anti-mouse or monoclonal rat anti-mouse antibodies or anti-human antibodies, capable of binding to the Fc-portions of the said antibodies, directed against the membrane structures; and
- 1.2. 1.2.1. mixing the target-cell-associating antibodies (murine or human)
15 which is attached to the said particles or beads, or attached to the beads pre-coated with anti-mouse or antihuman antibodies recognizing the Fc-portions of the target associating antibodies, with the cell suspension containing the target-cells, or,
1.2.2. mixing free target-cell-associating antibodies with the cell
20 suspension containing the target cells and incubate this mixture for 5-10 min to 2 h, preferably 30 min, at a temperature between 0C and 20C, preferably 4C under gentle rotation, and;
- 1.3. incubating the mixture of the cell suspension and target-associating antibodies attached to paramagnetic particles or beads (1.2.1.), or
25 paramagnetic particles or beads, precoated with anti-mouse or anti-human antibodies recognizing the Fc-portion of the target-associating antibodies, to the mixture of incubated free target associating antibody and cell suspension (1.2.2.), and incubating, for 5-10 min to 2h preferably 30 min, at a temperature between 0C and 25C, preferably
30 4C, under gentle rotation, and;
- 1.4. 1.4.1. if the target cell population is contained in blood or bone marrow aspirates the hydrophobic forces associated with antibody-coated particles are reduced by pre-incubating the antibody-coated

- particles and the cell suspension with mild detergents in suitable concentrations, e.g. Tween 20™ in concentrations less than 0.1% for 30 min at 4C, and/or;
- 5 1.4.2. by incubating the cell suspensions, untreated or pretreated with formalin, alcohol or other fixatives, with other antibodies or antibody fragments binding to extracellular or intracellular molecules present in the target cells and the antibodies used are labeled in advance by peroxidase, alkaline phosphatase, or other enzymes permitting visualization of the binding by addition and incubation with relevant
- 10 substrates, or;
- 1.4.3. the antibody fragments are biotinylated and the binding visualized when adding the incubating with avidin complexed to peroxidase, alkaline phosphatase, or other enzymes, with addition and incubation with relevant substrates, or;
- 15 1.5. subjecting the incubated paramagnetic particle-antibodies-cell mixture (1.3) to a magnetic field if the density of target-cells is low, or if the ratio of target cell/total cells in the cell mixture is low (< 1 %) and then
- 20 1.5.1. examining and counting the stained and unstained particle-target-cell complexes in the cell suspension, using a microscope and/or a suitable cell/particle counting device, or,
- 25 1.5.2. transferring the isolated target cell suspension to the cell filtering device or cell separator (20) in which the cell suspension is applied in the microwell, using membrane filter suitable to retain the particle/target-cell complexes, using or not using suction, removing the filters with the isolated target cells from the said cell filtering device to be fixed and stained by known immunohistochemical methods and viewed by microscope or adding a culture medium for the purpose of propagating the isolated target cell complexes situated on the filter to be charac-
- 30 terised for specific biochemical and biological features, or;
- 1.6. if the ratio of target-cells/total cells in the cell suspension is adequate (> 1 %) examining and counting the target-cells in the incubated mixture of paramagnetic particles, antibodies and cell mixture (1.3), or in the case when the antibodies or antibody fragments are conjugated

to non-paramagnetic particles that can be visualized directly because of colour or through enzymatic activation,

- 1.6.1. using a microscope and/or a suitable cell/particle counting device, or,
- 1.6.2. transferring the isolated target cell suspension to the cell filtering device or cell separator and perform the method according to 1.5.2. above.
- 5.
2. Method according to claim 1,
~~characterised~~ by directing the antibody or fragments thereof against the antigens in normal, living cells, such as liver hepatocytes, Kupffer cells and endothelial cells type 1 and 2 and Clara cells of the lung,
- 10 endothelial cells of specific organs, pancreatic exocrine and endocrine cells, kidney tubule cells, bladder epithelial cells, brain glial and ependymal cells, bladder and prostate epithelial cells, ciliated cells of airways, different subpopulations of mucosal cells in the gastrointestinal tract, pituitary cells, and other endocrine cells in various hormone producing organs.
- 15 3. Method according to one of the preceding claims,
~~characterised by~~ using as the said target-cell antibody an antibody which is reactive with antigens present on subpopulations of normal cells and oncogenic products expressed on the membrane of normal tissue cells.
- 20 4. Method according to one of the preceding claims,
~~characterised by~~ using as the said positive selecting antibody, an antibody which is directed against growth factor receptors on the membrane of normal cells, for example the EGF-receptor, PDGF (A and B) receptor, insulin receptors, insulin-like receptors transferrin receptor, NGF and FGF
- 25 receptors.
5. Method according to one of the preceding claims,
~~characterised by~~ using an antibody directed against the group of integrins and other adhesion membrane molecules, and MDR proteins in normal cells.

6. Method according to one of the preceding claims,
~~characterised by~~ directing the antibody or fragments thereof
against antigen or receptors in cells with abnormal developmental patterns,
preferably such as primary and metastatic cancer cells.
- 5 7. Method according to one of the preceding claims,
~~characterised by~~ using as the said target-cell associating
antibodies, antibodies of the IgG isotype, or F(ab')₂ or F(ab) fragments, or
IgM, or fragments of IgM.
- 10 8. Method according to one of the preceding claims,
~~characterised by~~ preparing the mentioned cell suspension from
mixed cell populations comprising mammalian tissues, for examples human
bone marrow and peripheral blood, from pleural and peritoneal effusions,
other body fluids, for example urine, cerebrospinal fluid, semen, lymph, or
from solid tumors in normal tissues and organs, for example liver, lymphatic
15 nodes, spleen, lung, pancreas, bone tissue, central nervous system, prostatic
gland, skin and mucous membranes.
9. Method according to one of the preceding claims,
~~characterised by that~~ the antibody or antibody fragments is
directed against groups of antigen determinants, such as those listed in Table
20 1 (see Appendix).
10. Method according to one of the preceding claims,
~~characterised by~~ using as the said target-cell antibody an
antibody or antibody fragment which is directed against growth factor
receptors and oncogene products expressed on the membrane of malignant
25 cells, for example insulin receptors, insulin-like receptors and FGF receptors
in addition to those listed in Table 1 (see Appendix).
11. Method according to one of the preceding claims,
~~characterised by~~ using an antibody or antibody fragment directed
against the group of integrins, other adhesion membrane molecules and MDR
30 proteins in abnormal cells as listed in Table 1 (see Appendix).

12. Method according to one of the preceding claims,
~~characterised in~~ that the used antibodies, antibody fragments or combinations of these are directed to the antigen determinants as listed in Table 1 (see Appendix).
- 5 13. Method according to one of the preceding claims,
~~characterised by~~ using as the said antibody an antibody which is reactive with antigens present on abnormal cells, for example breast, ovarian and lung carcinoma cells, melanoma, sarcoma, glioblastoma and cancer cells of the gastrointestinal and genitourinary tract, and of the reticuloendothelial
10 system, and/or target-cells associated with non-neoplastic diseases, such as cardiovascular, neurological, pulmonary, autoimmune gastrointestinal, genitourinary, reticuloendothelial and other disorders.
14. Cell filtering device or cell separator (20) for separating particle-target-cell complexes from unbound beads, unspecifically bound non-target cells and
15 unbound non-target cells in a cell suspension of mixed cell populations,
~~characterised by~~ comprising on filtrate collection box (22) with or without guiding pin(s) (28), with lid (21), with/without low pressure vacuum attachment part (23) containing a number of multiwell units (24) with/without guiding notch (29), with cell separator membrane filter (25) and
20 membrane support (25a) detachably fixed to the bottom of the multiwell unit (24).
15. Cell filtering device (20) according to claim 14,
~~characterised in~~ that the pores of said membrane filter are regular and consistent in shape and size, such as is found in for example
25 nylon monofilament membranes with pore sizes varying from $5\mu\text{m}$ to $75\mu\text{m}$, preferably $20\mu\text{m}$.
16. Use of the detection method according to one of the preceding claims, for isolation of target-cells, whereby the complex of cells and the paramagnetic particles are exposed to a magnetic field and the resulting
30 magnetically aggregated cells and/or cells isolated by using the cell filtering device (20) are further subjected to biological, biochemical and immunological examinations, including also characterisation of specific genes at the DNA, mRNA and protein level, including polymerase chain reaction (PCR) and reverse transcriptase PCR.

17. Use of the method for detection of specific target-cells according to one of the preceding claims, whereby it is established *in vitro* cell cultures from the separated paramagnetic/or non-magnetic particle-target-cell-complexes, and/or for inoculation into immunodeficient animals, preferably to establish human tumor xenografts in the said animals.
18. Kit for performing the method according to one of the preceding claims, characterised by that it comprises;
- 18.1 specific antibodies or antibody fragments directed to the antigen receptors on the wanted target-cells, where said antibody or antibody fragment is bound or can be bound to included paramagnetic particles, without removing their antigen-binding ability, and/or
- 18.2. paramagnetic particles precoated with specific anti-Fc antibodies, preferably polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-human antibodies, capable of binding to the Fc portions of the target-cell associating antibodies, and specific free target-cell antibodies, and/or
- 18.3. paramagnetic particles precoated with specific anti-Fc antibodies, preferably polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-human antibodies, capable of binding to the Fc-portions of the target-cell associating antibodies, bound to specific anti-target-cell antibodies, and/or
- 18.4. other specific antibodies or antibody fragments directed against antigens/receptors within or on the wanted target cells, where said antibodies or antibody fragments are conjugated to biotin, peroxidase, alkaline phosphatase, or other enzymes, or where said antibodies or antibody fragments are bound to non-paramagnetic particles with specific colours or with bound enzymes such as peroxidase and alkaline phosphatase, and/or
- 18.5. cell filter device according to claim 14 and 15.
- 18.6. paramagnetic or non-paramagnetic particles precoated with specific target cell antigen or group of antigens for use as a control or standard with or without use of the cell filter device.

1/5

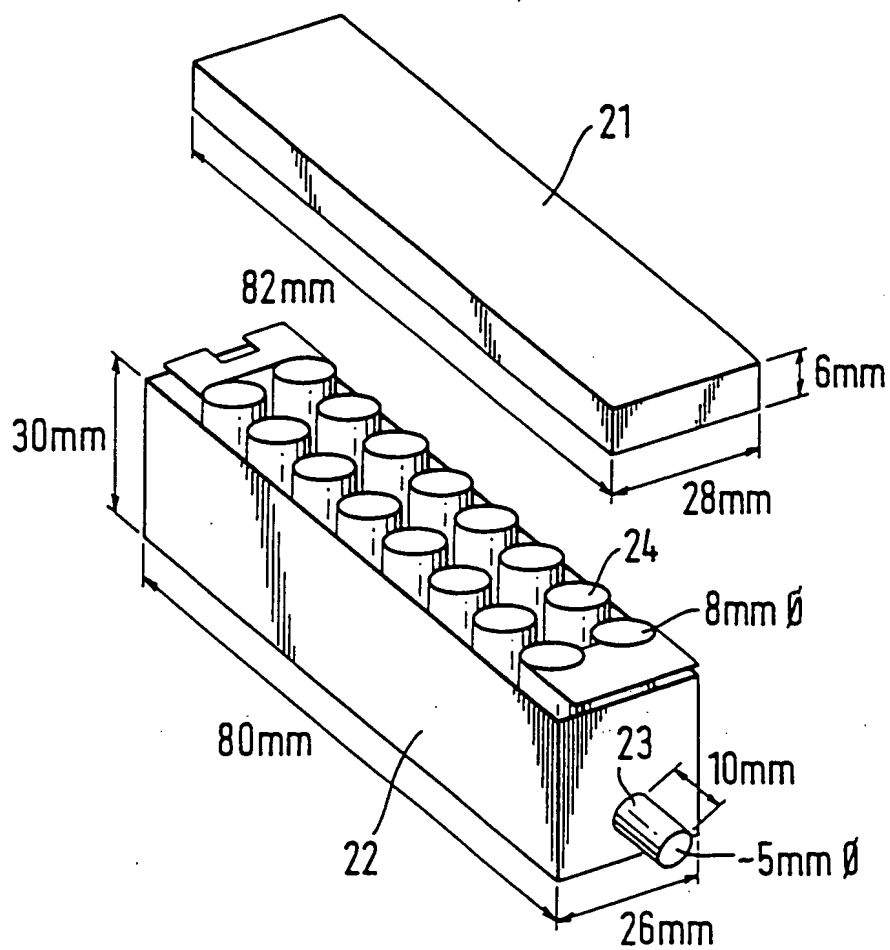


Fig. 1.1

2/5

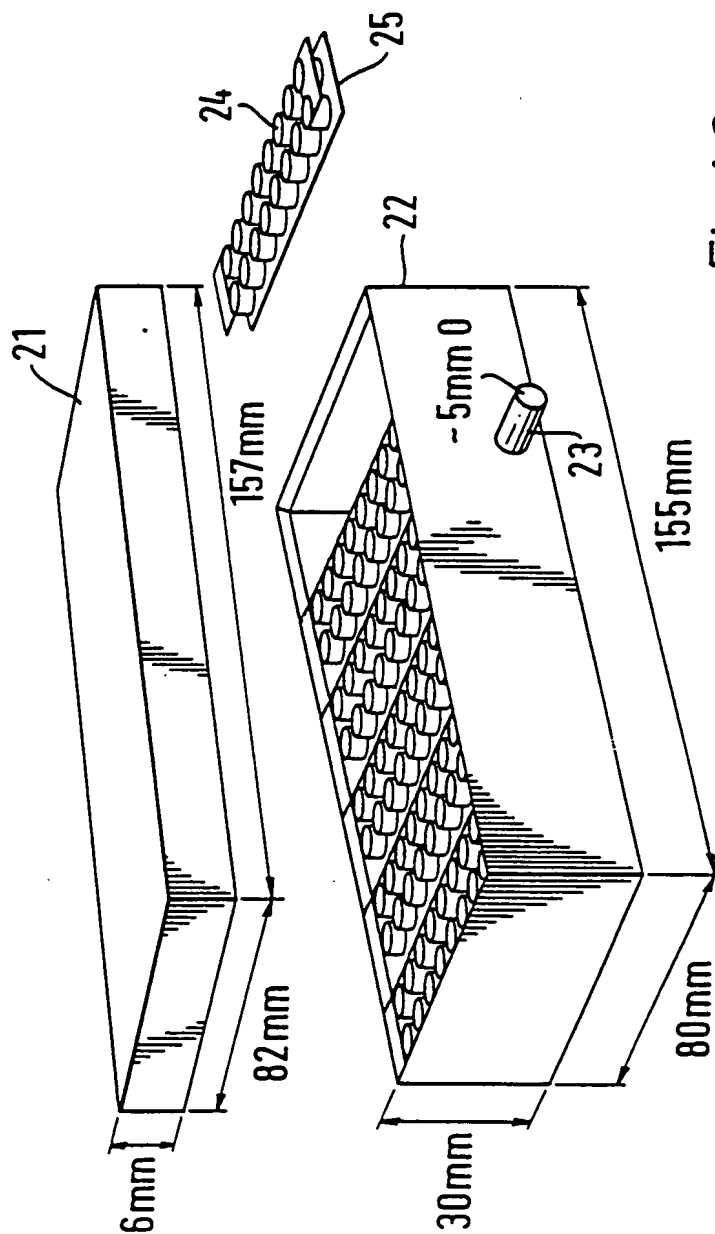


Fig. 1.2

3/5

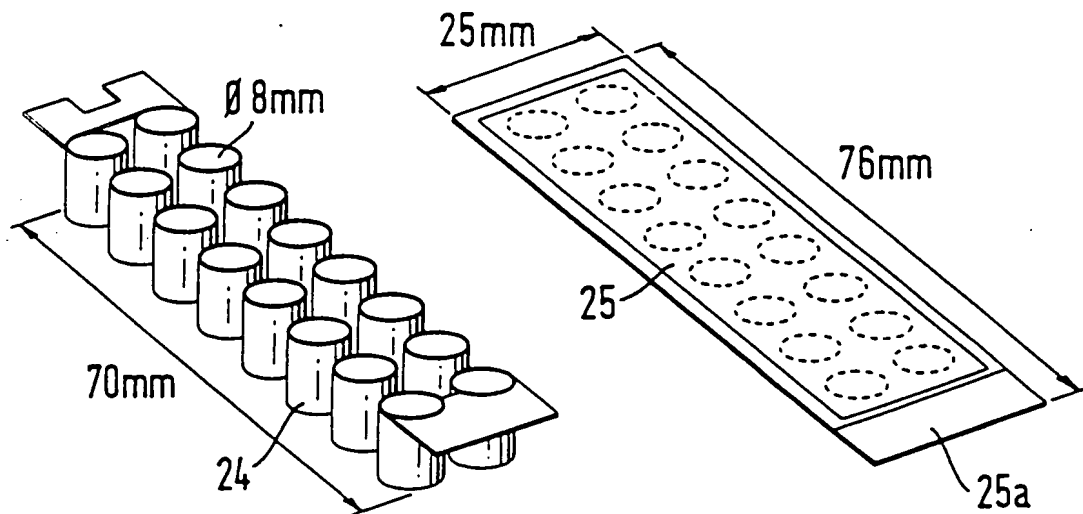
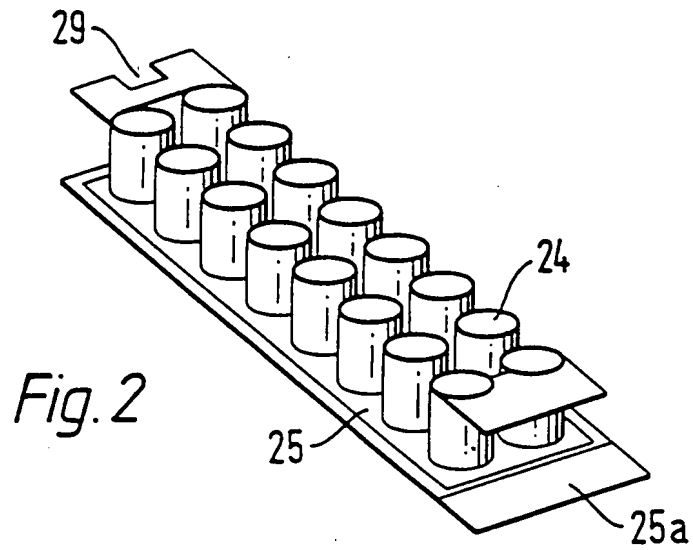


Fig. 3

4/5

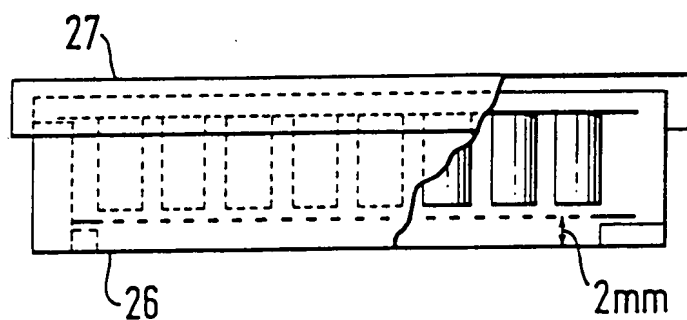
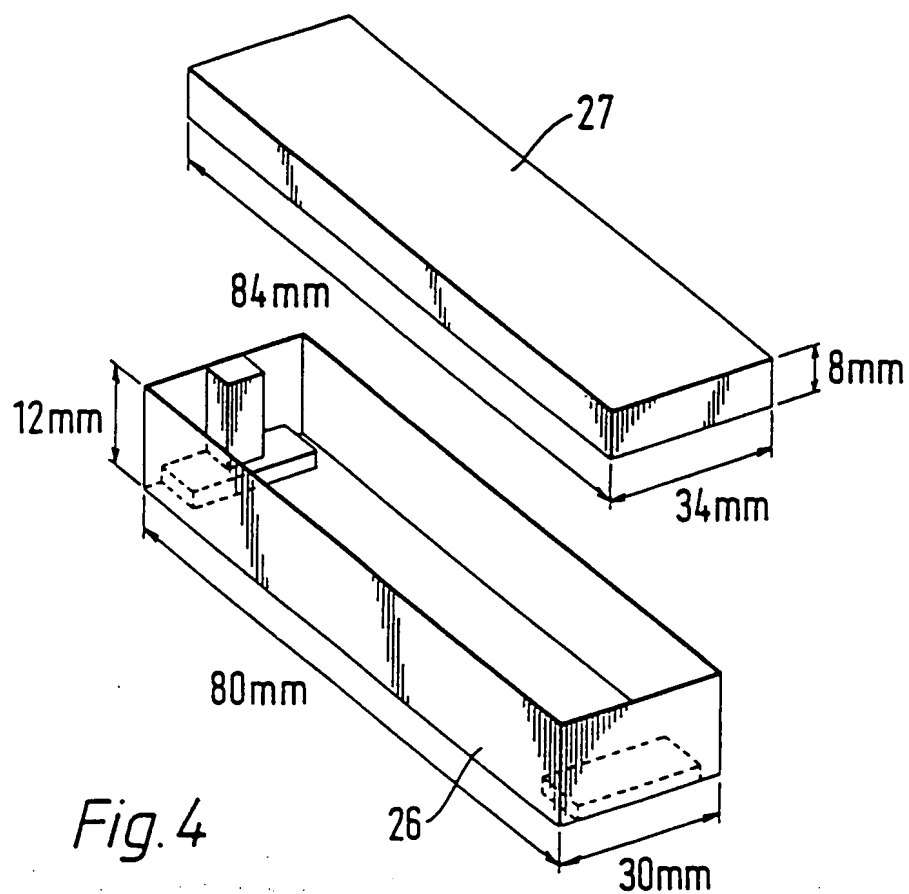


Fig. 4a

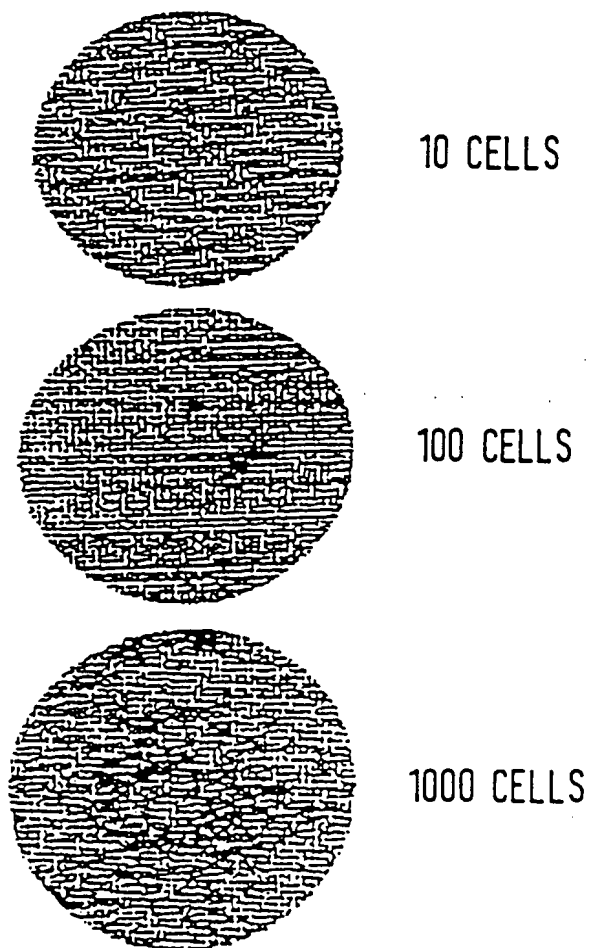
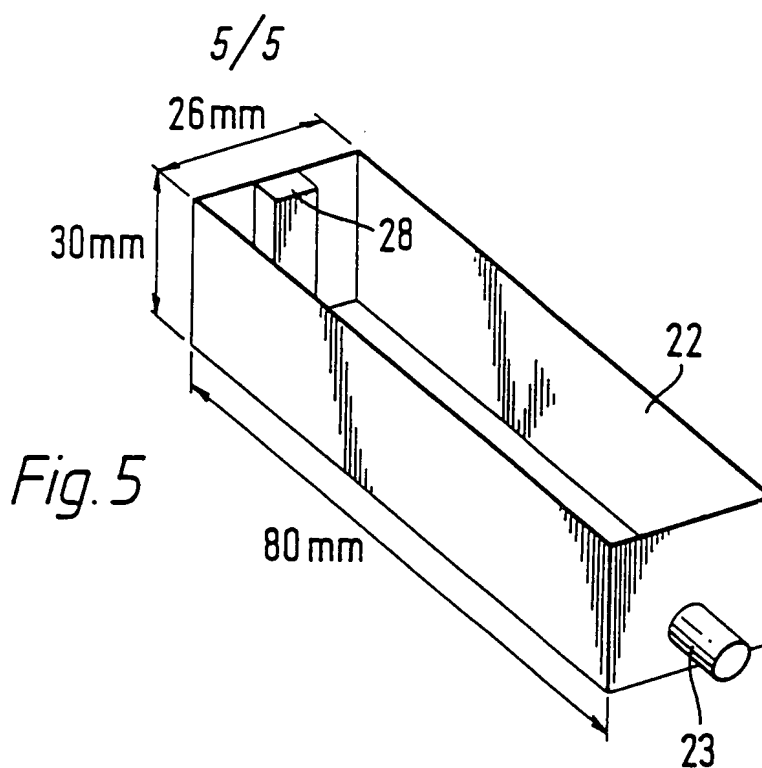


Fig. 6